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## METHODS IN CELL PHYSIOLOGY

# A novel human cell culture model to study visceral smooth muscle phenotypic modulation in health and disease

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**Vaes RD, van den Berk L, Boonen B, van Dijk DP, Olde Damink SW, Rensen SS.** A novel human cell culture model to study visceral smooth muscle phenotypic modulation in health and disease. *Am J Physiol Cell Physiol* 315: C598–C607, 2018. First published July 25, 2018; doi:10.1152/ajpcell.00167.2017.—Adaptation of the smooth muscle cell (SMC) phenotype is essential for homeostasis and is often involved in pathologies of visceral organs (e.g., uterus, bladder, gastrointestinal tract). In vitro studies of the behavior of visceral SMCs under (patho)-physiological conditions are hampered by a spontaneous, uncontrolled phenotypic modulation of visceral SMCs under regular tissue culture conditions. We aimed to develop a new visceral SMC culture model that allows controlled phenotypic modulation. Human uterine SMCs [ULTR and telomerase-immortalized human myometrial cells (hTERT-HM)] were grown to confluency and kept for up to 6 days on regular tissue culture surfaces or basement membrane (BM) matrix-coated surfaces in the presence of 0–10% serum. mRNA and protein expression and localization of SMC-specific phenotype markers and their transcriptional regulators were investigated by quantitative PCR, Western blotting, and immunofluorescence. Maintaining visceral SMCs confluent for 6 days increased  $\alpha$ -smooth muscle actin (1.9-fold) and smooth muscle protein 22- $\alpha$  (3.1-fold), whereas smooth muscle myosin heavy chain was only slightly upregulated (1.3-fold). Culturing on a BM matrix-coated surface further increased these proteins and also markedly promoted mRNA expression of  $\gamma$ -smooth muscle actin (15.0-fold), smoothelin (3.5-fold), h-caldesmon (5.2-fold), serum response factor (7.6-fold), and myocardin (8.1-fold). Whereas additional serum deprivation only minimally affected contractile markers, platelet-derived growth factor-BB and transforming growth factor  $\beta$ 1 consistently reduced versus increased their expression. In conclusion, we present a simple and reproducible visceral SMC culture system that allows controlled phenotypic modulation toward both the synthetic and the contractile phenotype. This may greatly facilitate the identification of factors that drive visceral SMC phenotypic changes in health and disease.

basal membrane matrix; differentiation; phenotypic modulation; visceral smooth muscle

## INTRODUCTION

The walls of many visceral organs are surrounded by well-developed smooth muscle layers that enable endured constriction or dilation, resulting in transport or storage of fluids. The smooth muscle cells (SMCs) within these layers possess the

unique ability to change their phenotype from a so-called contractile state toward a synthetic state. Whereas contractile SMCs are characterized by an elongated spindle-shaped morphology and high expression of specific contractile marker proteins (31), synthetic SMCs show a decreased expression of these contractile markers and an increased proliferation rate, high migration rate, and pronounced synthesis of extracellular matrix proteins (31).

SMC phenotypic plasticity is essential for adaptation of visceral organs to changes in local environmental conditions in health. For example, during pregnancy, uterine SMC hypertrophy and collagen synthesis are induced by ovarian hormones to cope with the stretch induced by the growing fetus and to maintain a quiescent contractile state until delivery (2, 15, 32). However, SMC phenotypic plasticity can also contribute to disease. An example is inflammatory bowel disease, where increased TNF- $\alpha$  and IL-1 $\beta$  levels reduce intestinal SMC contractility, contributing to intestinal motility problems (22–24). SMC hypertrophy and reduced smooth muscle contractility also underlie the significant increase in bladder mass during partial bladder outlet obstruction secondary to prostatic hyperplasia (3, 5, 42).

Given the importance of visceral SMC phenotypic plasticity for the pathogenesis of various disorders as well as normal physiological phenomena, it is pivotal to gain more mechanistic insight into the molecular processes involved and the relevant environmental cues. To this end, a visceral SMC culture system in which the SMC phenotype can be modulated in a controlled way is a prerequisite. Unfortunately, SMCs modulate uncontrollably toward a synthetic phenotype under standard cell culture conditions that lack the physiological microenvironment that normally keeps the cells in a contractile phenotype (6). In particular, a highly structured basal membrane (BM) matrix has been shown to be crucial for maintaining the contractile SMC phenotype (36). Additional elements of the cellular environment, including cell-cell interactions (18), humoral factors (e.g., growth factors, cytokines) (18), and mechanical forces, further regulate the SMC phenotype.

We here present the first visceral SMC culture system in which SMCs can be kept in either a highly contractile state or modulated toward the synthetic phenotype in a controlled way, developed by careful optimization of the growth surface, serum concentration, and cell-cell interaction. For the optimization of this culture model, we used well-characterized immortalized human uterine SMC lines that retain in vivo SMC characteristics, such as the expression of contractile and myometrial

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SMC markers (7, 28). This novel culture model can be used to further elucidate the molecular mechanisms driving SMC phenotypic switching and greatly facilitates the study of visceral SMC phenotypic modulation in health and disease.

## MATERIALS AND METHODS

**Cell culture conditions.** Human uterine SMCs (ULTR cells) (28) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO, cat. no. 42430) supplemented with 4 mM L-Glutamine, 10% (vol/vol) fetal bovine serum (FBS) (Greiner Bio-one, cat. no. 758093), and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin; GIBCO). Human myometrial SMCs immortalized with human telomerase reverse transcriptase (hTERT-HM) were a gift from Dr. Jennifer Condon (7). These cells were maintained in DMEM/F12 (GIBCO, cat. no. 31331) supplemented with 10% FBS, 10 mM HEPES (GIBCO, cat. no. 15630), and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin). All experiments were performed within 8 passages, and both cell lines were maintained at 37°C, 5% CO<sub>2</sub> in a humidified incubator.

For experiments, SMCs were plated at a density of  $1 \times 10^4$  cells/cm<sup>2</sup> on either standard tissue culture (TC)-treated (Eppendorf) or BM matrix-coated (Geltrex Reduced Growth Factor Basement Membrane Matrix, GIBCO, cat. no. A14132) surfaces. To obtain BM-coated plates, tissue culture-treated multiwell polystyrene plates (Eppendorf) were coated with 100 µl/cm<sup>2</sup> ice-cold Geltrex (1:50 in DMEM without supplements) and incubated for 2 h at 37°C before plating cells. Subsequently, when cells reached >90% confluency (after 48 h), regular culture medium used for maintaining the cells was replaced by advanced DMEM/F-12 (GIBCO, cat. no. 12634) that allows culturing cells with reduced serum supplementation due to the addition of specific components, including ethanolamine, glutathione, ascorbic acid, insulin, transferrin, and AlbuMAX lipid-rich bovine serum albumin. Advanced DMEM/F-12 medium was supplemented with different serum concentrations (10%, 2%, or 0% FBS), 1% (vol/vol) GlutaMAX (GIBCO, cat. no. 35050), and antibiotics (50 U/ml penicillin and 50 µg/ml streptomycin; GIBCO). In indicated experiments, SMCs were treated with either PDGF-BB (30 ng/ml; PeproTech, cat. co. 100-14B) or transforming growth factor β1 (TGF-β1) (5 ng/ml; PeproTech, cat. no. 100-21) to induce phenotypic modulation. Medium was refreshed every 3 days.

**Quantitative real-time PCR.** Total RNA was isolated using TRI Reagent (Sigma, St. Louis, MO) according to the manufacturer's protocol. RNA yield was measured with a DeNovix DS-11 spectrophotometer. A total of 750 ng RNA were reversed transcribed to cDNA using the SensiFast cDNA Synthesis Kit according to the manufacturer's instructions (Bioline GmbH, Germany).

To quantify mRNA expression levels of SMC phenotype markers, quantitative real-time PCR (qRT-PCR) analysis was performed on the LightCycler480 (Roche) using a three-step PCR program followed by melting curve analysis. cDNA was amplified with the SensiMix SYBR Hi-Rox Kit (Bioline, cat. no. QT605-05). Specific primer pairs for each gene were ordered from Sigma and are listed in Table 1. Relative gene expression levels were derived from the LinRegPCR (version 2016.1) method (33) and normalized to the geometric average of two reference genes, cyclophilin A (*CYP*A) and β-2-microglobulin (*B2M*).

**Western blotting.** Cells were harvested with lysis buffer containing 10 mM Tris, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 10% glycerol, 0.1% (wt/vol) SDS, and 0.5% (vol/vol) sodium deoxycholate supplemented with protease inhibitor cocktail tablets (Roche, Mannheim, Germany). Whole cell lysates were incubated on ice for 30 min and subsequently centrifuged at 16,000 g for 30 min at 4°C. Supernatant was transferred to a new tube, and total soluble protein concentrations were determined using the Pierce BCA Protein Assay Kit (Thermo Fisher, Rockford, IL). Laemmli sample buffer [ $\times 4$ ; 250 mM Tris-HCl, pH 6.8, 8% (wt/vol) SDS, 40% (vol/vol)

Table 1. *Quantitative RT-PCR primers*

Gene	Forward Primer (5'→3')	Reverse Primer (5'→3')
<i>CYP</i> A	CTCGAATAAGTTTGACTTGTGTTT	CTAGGCATGGGAGGGAACA
<i>B2M</i>	TCCATCCGACATTGAAGTTG	CGGCAGGCATACTCATCTT
<i>ACTA2</i>	CCGGGACTAAGACGGGAATC	TTGTACACACCAAGGCAGT
<i>ACTG2</i>	CGCCCTCGCCACCAAG	CCTTGGGATTAGGGGAGCC
<i>TAGLN</i>	AATTGATGGAACACCGGG	GGGAAAGCTCCTTGGAAGT
<i>SMTN</i>	ACCATCGAGATCAAGGACGG	CCAGTGTCTCTCTGCCCTC
<i>CALD1</i>	GCTGTCTAAAGAAACAGGGAG	GGTTGTCTCTCTGGAGCTT
<i>MYH11</i>	CATCTACGCCATCGCAGACA	CAGACTCGCCTGTGCATAGAA
<i>MYOCD</i>	GGGTCTGAGCATTCCTTGCT	CTGGACGTTTCAGTGGTGT
<i>SRF</i>	TTGCTGAGTGAAGGGGCCTA	AGTTTTCGGGTGGCAAGGT

*ACTA2*, α-smooth muscle actin; *ACTG2*, γ-smooth muscle actin; *B2M*, β-2-microglobulin; *CALD1*, caldesmon 1 (primers specific for heavy caldesmon transcript); *CYP*A, cyclophilin A; *MYH11*, myosin heavy chain 11; *MYOCD*, myocardin; *SMTN*, smoothelin; *SRF*, serum response factor; *TAGLN*, smooth muscle protein 22-α.

glycerol, 355 mM 2-Mercaptoethanol, and 0.02% (wt/vol) bromophenol blue] was added to the lysate, followed by heating the samples for 5 min at 95°C. Equal amounts of protein (10 µg) were loaded per lane on a 4%–15% Criterion TGX Stain-Free precast Gel (Bio-Rad). Following electrophoretic separation, the proteins were transferred onto a Transblot Turbo polyvinylidene difluoride transfer membrane (Bio-Rad) by electro-blotting on the Trans-Blot Turbo Blotting System (Bio-Rad). After blocking with 5% (wt/vol) BSA (Lampire Biological Laboratories, cat. no. 7500804), membranes were incubated overnight at 4°C with specific monoclonal primary antibodies directed against anti-smooth muscle myosin heavy chain (anti-SM-MHC) (1:1,000, Clone BT-562, Alfa Aesar, cat. no. J64817AMJ), anti-α-smooth muscle actin (anti-α-SMA) (1:1,000, Clone 1A4, DAKO, cat. no. M0851), and anti-smooth muscle protein 22-α (anti-SM22α) (1:1,000) (the mouse anti-SM22α hybridoma cell line, clone 3E11, was a kind gift from Dr. A. Chiavegato, University of Padua, Italy). After washing with Tris-buffered saline-Tween 20 (0.01%), the blots were probed for 1 h with an appropriate peroxidase-conjugated secondary antibody (Vector Laboratories), and signals were visualized using SuperSignal West Pico chemiluminescent substrate (Thermo Scientific) according to the manufacturer's instructions. Images were obtained with a molecular imager (Amersham Imager 600, GE Healthcare Life Sciences), and total band intensity was quantified with ImageQuant TL software (v8.1.0.0, GE Healthcare Life Sciences).

**Immunofluorescence.** For double immunofluorescence staining, uterine SMCs were cultured on 18-mm glass coverslips (Thermo Scientific). Cells were fixed for 5 min in 50% (vol/vol) methanol/50% (vol/vol) acetone at −20°C. Subsequently, cells were rinsed in Dulbecco's PBS (GIBCO, Life Technologies), followed by a 60-min incubation with mouse monoclonal IgG2a antibody recognizing anti-α-SMA (1:200, Clone 1A4, DAKO) and mouse monoclonal IgG1 antibody recognizing SM22α (1:150, Clone 3E11). Alexa Fluor 488-conjugated goat anti-mouse IgG1 (Invitrogen, cat. no. A-21121) and Alexa Fluor 594-conjugated goat anti-mouse IgG2a (Invitrogen, cat. no. A-21135) were used as secondary antibodies. Nuclei were counterstained by 4',6'-diamidino-2-phenylindole. Coverslips were mounted in fluorescent mounting medium (DAKO). Digital images were taken using a Leica TCS SP8 X automated inverted confocal microscope equipped with an HC PL APO CS2 63x/1.40 oil objective and processed using ImageJ (version 1.51h).

**Statistical analysis.** Data are obtained from two or three independent experiments, and results are expressed as mean ± SE. Raw data was entered in IBM SPSS 24 for Microsoft Windows, and statistical analyses were performed using the nonparametric Mann-Whitney U-test to compare differences between two groups. In case of more than two groups, the nonparametric Kruskal-Wallis test was applied, followed by Dunn's post hoc testing with Bonferroni correction. A *P* value of *P* < 0.05 was considered statistically significant.

## RESULTS

**Effect of prolonged cell-cell contact on SMC-specific contractile markers.** Reduced cell-cell contact as a consequence of culturing SMCs at a low density has previously been shown to result in a decreased expression of contractile marker proteins (18). At confluence, vascular SMCs lose their proliferative capacity and concurrently increase their expression of a panel of SMC-specific contractile proteins like  $\alpha$ -SMA, SM22 $\alpha$ , and SM-MHC. To investigate whether visceral SMCs exhibit a similar cellular behavior, we cultured ULTR cells toward a confluent state and maintained them for 3 or 6 days in the presence of 10% FBS before analyzing contractile marker expression. These immortalized cells have previously been shown to acquire characteristics of the synthetic SMC phenotype when cultured toward a subconfluent state, including a high proliferation rate, decreased cell size, and irregular distribution of  $\alpha$ -actin filaments (28). Culturing ULTR SMCs for 3 days in a confluent state resulted in slightly increased SM-MHC and SM22 $\alpha$  protein levels, but these differences were not significant (Fig. 1A). However, contractile protein levels continued to increase; after 6 days, SM22 $\alpha$  protein levels were significantly increased by 3.1-fold ( $P = 0.01$ ), and  $\alpha$ -SMA protein levels tended to be significantly increased (1.9-fold,  $P = 0.09$ ). These protein data indicate that increasing cell-cell contact only minimally promotes the contractile phenotype in visceral SMCs. However, mRNA expression of many contractile marker genes was significantly increased after 6 days (Fig. 1B): *ACTA2* (5.3-fold,  $P < 0.001$ ), *ACTG2* (1.3-fold,  $P < 0.001$ ), *CALD1* (2.2-fold,  $P = 0.03$ ), and

*MYH11* (2.2-fold,  $P = 0.04$ ). This suggests that increased cell-cell contact initiates transcriptional upregulation of contractile genes, although this was not sufficient to induce differences at the protein level. Nevertheless, transcription of serum response factor (*SRF*) and myocardin (*MYOCD*), genes important for transcriptional regulation of the contractile phenotype markers, was not induced by prolonged confluency (Fig. 1C).

**Basement membrane components promote the visceral SMC contractile phenotype.** Since the interaction of cells with the extracellular microenvironment is known to influence vascular SMC differentiation, migration, proliferation, and survival (30), we next investigated whether the addition of basal membrane matrix (BM) components could further stimulate the expression of contractile proteins in visceral SMCs. In comparison with cells grown on TC-coated surfaces (Fig. 1), SMCs cultured on a BM-coated surface showed a higher increase of *ACTA2* gene expression after 6 days of culturing at confluency (TC: 2.9-fold,  $P < 0.001$  vs. BM: 8.2-fold,  $P = 0.002$ ) (Fig. 2A). More importantly, cells grown on BM markedly upregulated their expression of the more advanced contractile phenotype markers (*ACTG2*, 15.0-fold,  $P = 0.002$ ; *TAGLN*, 3.2-fold,  $P = 0.7$ ; *SMTN*, 3.5-fold,  $P = 0.002$ ; *CALD1*, 5.2-fold,  $P = 0.004$ ) (Fig. 2A) compared with the SMCs grown on TC-coated surfaces (Fig. 1B). These inductions were also observed at the protein level, where expression of  $\alpha$ -SMA was increased by 3.9-fold ( $P < 0.001$ ) and expression of SM22 $\alpha$  by 4.8-fold ( $P < 0.001$ ) (Fig. 2B). In addition, SM-MHC protein levels were significantly enhanced in cells grown on BM matrix

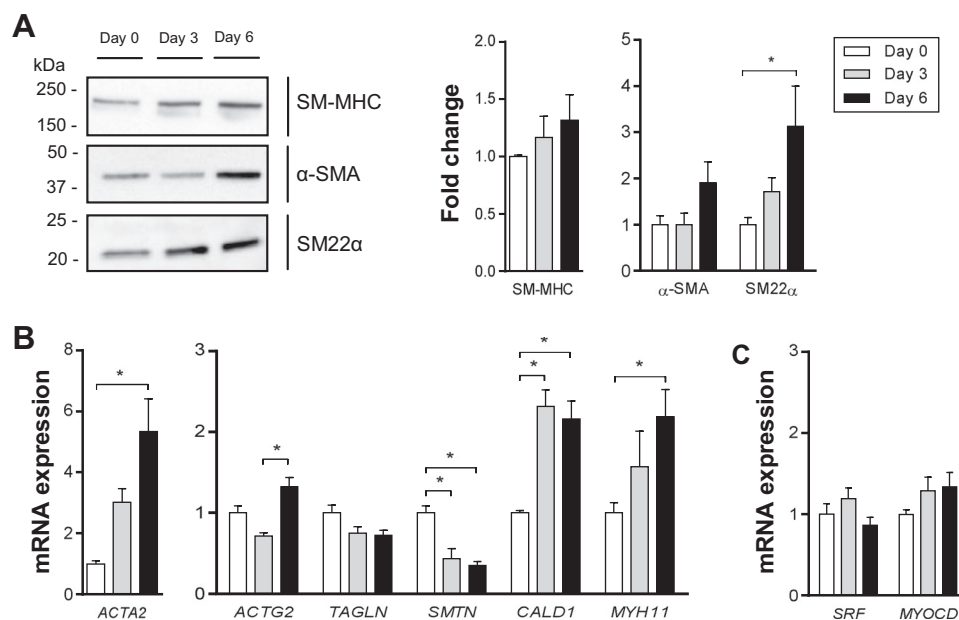
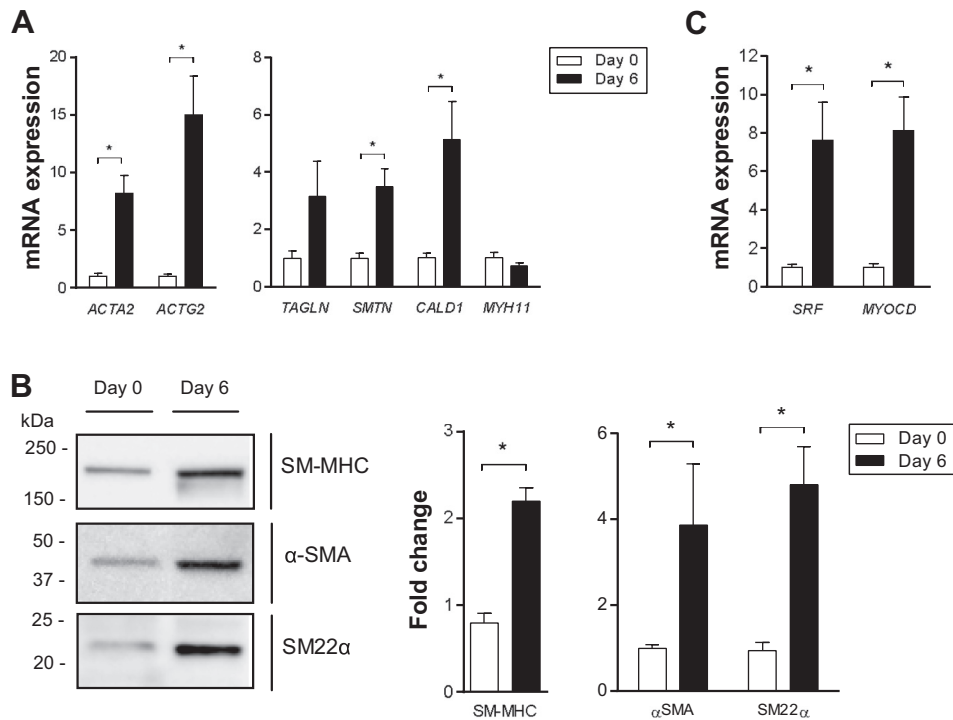


Fig. 1. Expression of smooth muscle cell (SMC)-specific contractile markers in confluent visceral SMCs. Visceral SMCs (ULTR cells) were cultured on a tissue culture (TC)-coated surface in medium containing 10% FBS. A: protein expression of smooth muscle myosin heavy chain (SM-MHC),  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA), and smooth muscle protein 22- $\alpha$  (SM22 $\alpha$ ) was determined by Western blot analysis. All samples from each independent experiment (SM-MHC,  $n = 6$ ;  $\alpha$ -SMA and SM22 $\alpha$ ,  $n = 9$ ) were derived at the same time and processed in parallel. B and C: mRNA expression levels of contractile (*ACTA2*, *ACTG2*, *TAGLN*, *SMTN*, *CALD1*, and *MYH11*) (B) and transcriptional regulatory (*SRF*, *MYOCD*) (C) genes were determined and are expressed relative to expression levels at day 0. Data were normalized to *CYPB* and *B2M* reference genes. Results from each time point were obtained from two (*CALD1* and *MYH11*;  $n = 6$ ) or three (*ACTA2*, *ACTG2*, *TAGLN*, *SMTN*, *SRF*, and *MYOCD*;  $n = 9$ ) independent experiments and are presented as mean  $\pm$  SE (\* $P < 0.05$  compared with D0, Kruskal-Wallis test followed by Dunn's post hoc testing with Bonferroni correction). *ACTA2*,  $\alpha$ -smooth muscle actin; *ACTG2*,  $\gamma$ -smooth muscle actin; *B2M*,  $\beta$ -2-microglobulin; *CALD1*, caldesmon; *CYPB*, cyclophilin B; *MYH11*, smooth muscle myosin heavy chain; *MYOCD*, myocardin; *SMTN*, smoothelin; *SRF*, serum response factor; *TAGLN*, smooth muscle protein 22- $\alpha$ .





**Fig. 2.** Culturing visceral smooth muscle cells (SMCs) on a basement membrane (BM) matrix-coated surface promotes a contractile phenotype. Visceral SMCs (ULTR) were seeded on a BM-coated growth surface in medium containing 10% FBS. **A** and **B**: mRNA expression levels of contractile (*ACTA2*, *ACTG2*, *TAGLN*, *SMTN*, *CALD1*, and *MYH11*) (**A**) and transcriptional regulatory (*SRF*, *MYOCD*) (**B**) genes were determined and are expressed as relative expression levels with *day 0* samples set at 1. Data were normalized to *CYPA* and *B2M* reference genes. **C**: protein expression levels of smooth muscle myosin heavy chain (SM-MHC), α-smooth muscle actin (α-SMA), and smooth muscle protein 22-α (SM22α) (**C**) were determined by Western blot analysis. All samples from each independent experiment were derived at the same time and processed in parallel (SM-MHC,  $n = 6$ ; α-SMA and SM22α,  $n = 9$ ). Results are presented as mean  $\pm$  SE (Mann-Whitney *U*-test, \* $P < 0.05$ ). *ACTA2*, α-smooth muscle actin; *ACTG2*, γ-smooth muscle actin; *B2M*, β-2-microglobulin; *CALD1*, caldesmon; *CYPA*, cyclophilin A; *MYH11*, smooth muscle myosin heavy chain; *MYOCD*, myocardin; *SMTN*, smoothelin; *SRF*, serum response factor; *TAGLN*, smooth muscle protein 22-α.

(2.2-fold,  $P = 0.002$ ). Moreover, mRNA expression of transcriptional regulators of the contractile phenotype was highly upregulated in SMCs cultured on BM matrix (*SRF*: 7.6-fold,  $P = 0.002$ ; *MYOCD*: 8.1-fold,  $P = 0.002$ ) (Fig. 2C).

**Effect of serum concentrations on the phenotype of SMCs grown on BM matrix.** Growth factors present in FBS are known to stimulate cell proliferation, migration, and matrix protein synthesis, which are all characteristics of the synthetic SMC phenotype. We therefore investigated whether reducing serum concentrations could further promote the contractile phenotype of visceral SMCs grown on BM matrix for 6 days. In comparison with the 10% FBS condition, only α-SMA protein levels were slightly increased (1.3-fold, Fig. 3A) when cells were cultured in 2% or 0% FBS, although these increases were not significant. However, SM-MHC and SM22α protein concentrations were similar when cells were grown in 10% versus 2% FBS (Fig. 3A), and complete serum deprivation even caused significantly decreased SM-MHC (0.7-fold,  $P = 0.03$ ) and SM22α protein expression (0.4-fold,  $P = 0.005$ ) (Fig. 3A). Of note, complete serum deprivation seemed to reduce cell viability, as indicated by cell morphology and the appearance of floating cells (data not shown). At the mRNA level, reducing serum concentrations did not increase expression of contractile phenotype markers in comparison with the 10% FBS condition (Fig. 3B), except for *CALD1*, which was slightly upregulated. This suggests that the known inhibitory effect of the growth factors on the SMC contractile phenotype is blunted when cells are grown on a BM matrix.

**Visceral SMCs undergo morphological and structural changes within an optimized SMC culture environment.** The results presented above indicate that by optimizing culture conditions, visceral SMCs maintain a relatively contractile phenotype characterized by high contractile marker protein expression. Consistent with this, the SMCs cultured at these optimized conditions underwent the typical morphological

changes that SMCs undergo during acquisition of a contractile phenotype. Whereas a heterogeneous population of rhomboid- and elongated spindle-shaped cells was present at *day 0*, cells cultured on basement membrane matrix proteins acquired the typical contractile phenotype-associated elongated spindle-shaped morphology upon 6 days of culturing (Fig. 4A). These morphological changes were accompanied by pronounced rearrangements of the contractile apparatus; α-SMA filaments in synthetic rhomboid SMCs ran crisscross through the cytoplasm (Fig. 4B, *a–c*), whereas they reorganized to run parallel to the long axis in elongated spindle-shaped SMCs (Fig. 4B, *d–f*). Moreover, SM22α was abundantly present along the actin filaments in contractile SMCs (Fig. 4B, *d–f*) compared with synthetic SMCs, in which SM22α was only marginally expressed and appeared to show less colocalization with α-SMA filaments (Fig. 4B, *a–c*).

**The contractile phenotype of visceral SMCs grown on BM can be modulated in a controlled way.** To further show the potential of this culture system for modeling disease states characterized by SMC phenotypic changes, we stimulated the cells with factors known to influence the vascular SMC phenotype (PDGF-BB and TGF-β1). In vascular SMCs, PDGF-BB has been shown to suppress the contractile phenotype, whereas TGF-β1 stimulates it (8, 13). PDGF-BB and TGF-β1 stimulation greatly influenced both protein and gene expression levels of contractile SMC markers of visceral SMCs cultured for 6 days on a BM-coated growth surface with 2% FBS. SMCs incubated for 48 h with PDGF-BB showed reduced protein levels of α-SMA (0.5-fold,  $P = 0.048$ ), and SM22α (0.6-fold,  $P = 0.4$ ) tended to be decreased as well (Fig. 5A). Conversely, stimulation with TGF-β1 further increased protein expression of both α-SMA (2.3-fold,  $P = 0.048$ ) and SM22α (1.9-fold,  $P = 0.04$ , Fig. 5A) in cells that already had a contractile phenotype as a result of culturing on BM matrix. Although protein levels of α-SMA and SM22α were altered

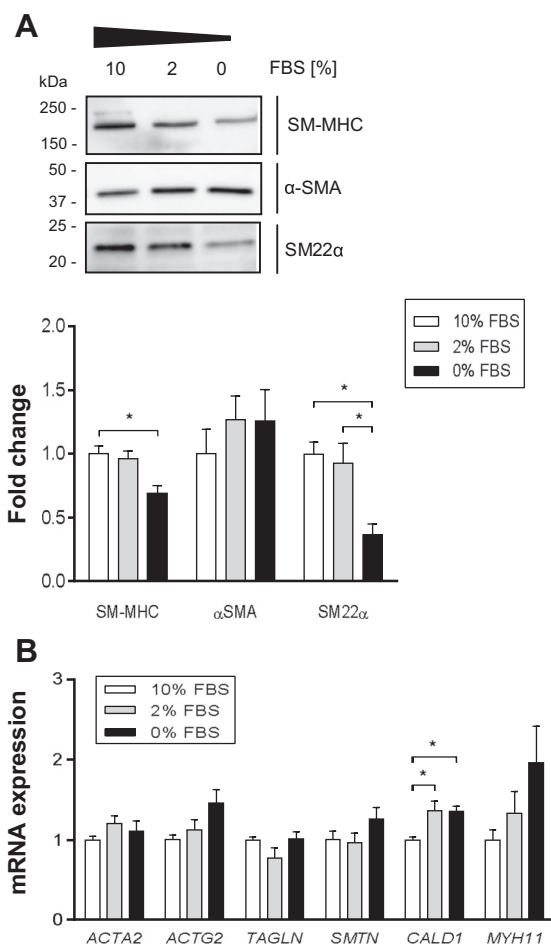


Fig. 3. Effect of serum on contractile marker expression in visceral smooth muscle cell (SMCs) grown on basement membrane (BM). Visceral SMCs (ULTR cells) were cultured on a BM-coated growth surface. When cells reached >90% confluency, growth medium was replaced by differentiation medium containing reduced serum concentrations (10%, 2%, or 0% FBS) for 6 days. **A**: protein expression levels of smooth muscle myosin heavy chain (SM-MHC),  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA), and smooth muscle protein 22- $\alpha$  (SM22 $\alpha$ ) were determined by Western blot analysis. All samples from each independent experiment were derived at the same time and processed in parallel (SM-MHC,  $n = 6$ ;  $\alpha$ -SMA and SM22 $\alpha$ ,  $n = 9$ ). **B**: mRNA expression levels of *ACTA2*, *ACTG2*, *TAGLN*, *SMTN*, *CALD1*, and *MYH11* were determined and are expressed as relative expression with day 0 samples set at 1. Data were normalized to *CYPA* and *B2M* reference genes. Results from each time point were obtained from two (*CALD1*, *MYH11*,  $n = 6$ ) or three (*ACTA2*, *ACTG2*, *TAGLN*, and *SMTN*;  $n = 9$ ) independent experiments and are presented as mean  $\pm$  SE (Kruskal-Wallis test followed by Dunn's post hoc testing with Bonferroni correction,  $*P < 0.05$ ). *ACTA2*,  $\alpha$ -smooth muscle actin; *ACTG2*,  $\gamma$ -smooth muscle actin; *B2M*,  $\beta$ -2-microglobulin; *CALD1*, caldesmon; *CYPA*, cyclophilin A; *MYH11*, smooth muscle myosin heavy chain; *SMTN*, smoothelin; *TAGLN*, smooth muscle protein 22- $\alpha$ .

after PDGF-BB and TGF- $\beta$ 1 stimulation, no differences in SM-MHC protein levels were observed. On the other hand, *MYH11* mRNA levels were markedly induced by TGF- $\beta$ 1 treatment, along with induction of *ACTG2*, *TAGLN*, *SMTN*, *SRF*, and *MYOCD* (Fig. 5, *B* and *C*). Treatment with PDGF-BB provoked significant reductions in *ACTA2*, *CALD1*, and *SRF* levels, as well as smaller, nonsignificant reductions of *ACTG2*, *TAGLN*, *MYH11*, and *MYOCD*. Overall, these data not only indicate that visceral SMCs can be kept in a contractile phenotype by culturing them on BM matrix for a prolonged time,

but also show that their phenotype can still be modulated in a controlled way toward both a more contractile and a more synthetic state.

**Validation of culture conditions for controlled phenotypic modulation of visceral smooth muscle cells.** To demonstrate that the culture conditions defined above can also be applied to study phenotypic modulation in other visceral SMCs, we validated our culture system using an independent uterine smooth muscle cell line (hTERT-HM). These highly proliferative, immortalized SMCs have been shown to retain smooth muscle-specific markers (e.g.,  $\alpha$ -SMA, *h*-caldesmon, and calponin) and display an elongated phenotype, potentially indicating a more contractile phenotype compared with ULTR cells (7). We first compared the morphology of confluent hTERT-HM cells grown on either TC- or BM-coated surfaces (Fig. 6*A*). SMCs cultured on TC-coated surfaces adopted the typical rhomboid-shaped morphology after 6 days, indicative of the synthetic phenotype. In accordance with previous results obtained using ULTR cells, we observed a homogeneous population of elongated spindle-shaped cells when hTERT-HM SMCs were cultured on BM-coated surfaces, consistent with modulation toward the contractile phenotype. Next, we validated the changes in protein expression levels of SMC-specific contractile markers upon culturing on TC-coated versus BM-coated surfaces (Fig. 6*B*). Similar to what was observed for ULTR SMCs, hTERT-HM cells cultured on BM-coated surfaces showed a marked upregulation of SM-MHC (2.1-fold,  $P < 0.001$ ),  $\alpha$ -SMA (4.2-fold,  $P < 0.001$ ), and SM22 $\alpha$  (2.3-fold,  $P < 0.001$ ) protein compared with hTERT-HM cells cultured on TC-coated surfaces. Finally, we investigated whether contractile hTERT-HM SMCs grown on BM were responsive to PDGF-BB and TGF- $\beta$ 1 to stimulate phenotypic modulation. Although these cells only slightly decreased gene and protein expression levels of SMC-specific contractile markers after PDGF-BB treatment, significantly increased levels of these markers were observed after stimulation with TGF- $\beta$ 1 (Fig. 6, *C–E*). Altogether, these results corroborate the data obtained with the ULTR cells and demonstrate that the culture conditions defined above can be applied to study phenotypic modulation in other visceral SMC lines.

## DISCUSSION

The present study describes culture conditions that can be applied to keep visceral SMCs in a highly contractile state while still allowing phenotypic modulation toward either a more contractile or synthetic phenotype in a controlled way. This is the first study in which the phenotypic response of visceral SMCs to combinations of microenvironmental factors, including cell-cell interactions, cell-BM interactions, and serum concentrations, was systematically investigated. Whereas serum deprivation did not significantly affect the SMC phenotype, increasing cell-cell interactions and cell-BM interactions greatly promoted the acquisition and maintenance of a contractile phenotype.

The vast majority of previous studies on visceral SMC phenotypic modulation used primary cells, which have important drawbacks since they have a limited life span and display uncontrolled phenotypic modulation toward the synthetic phenotype during the initial stages of culturing (6, 38). Together



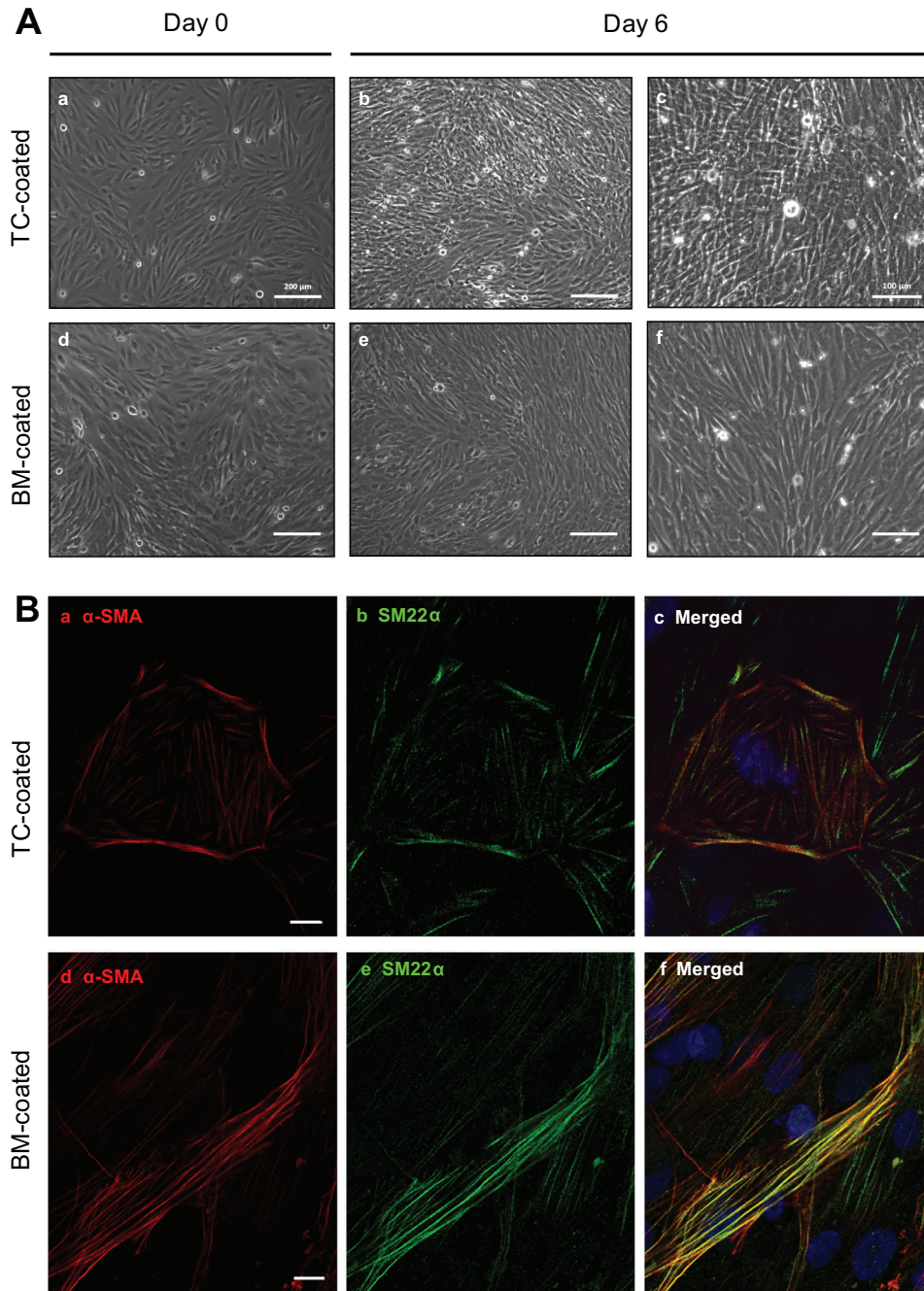


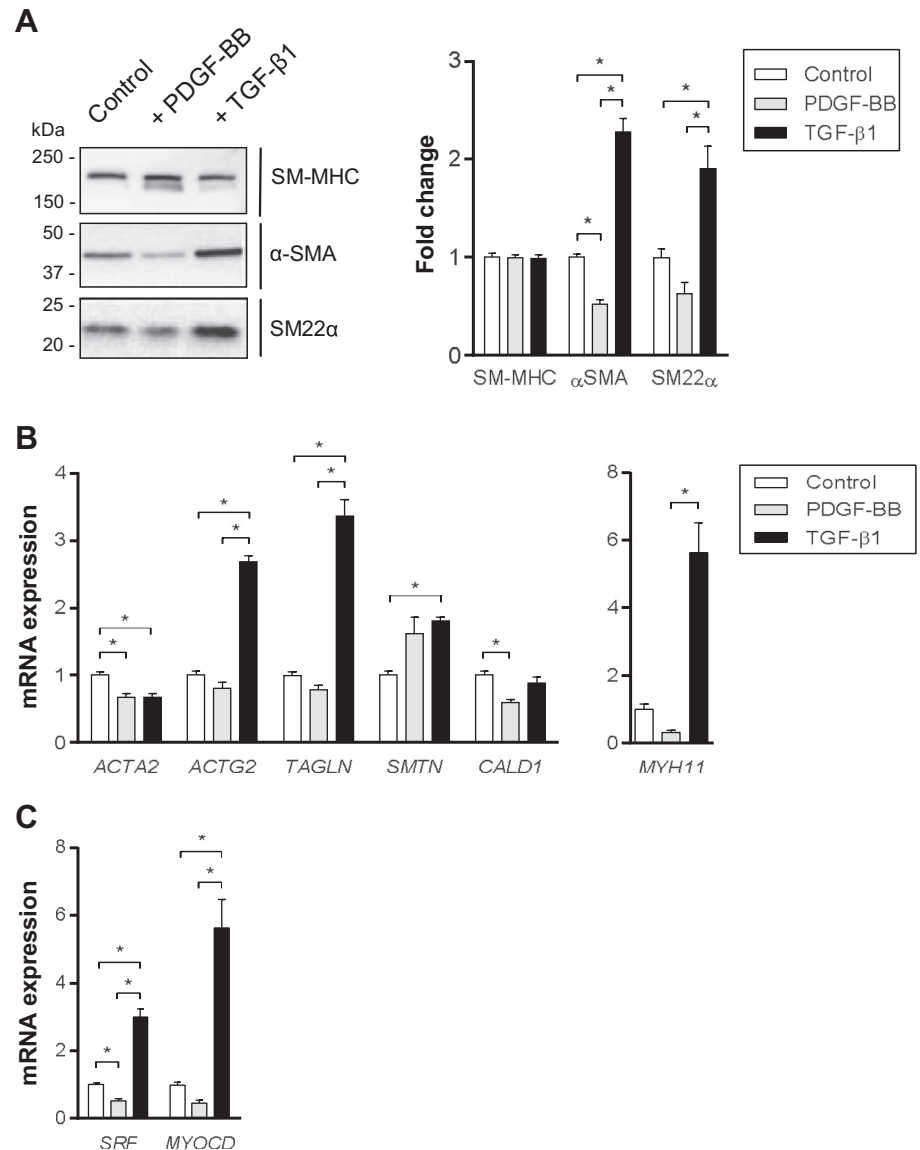
Fig. 4. Controlled modulation of morphological and structural characteristics of visceral smooth muscle cell (SMCs). Visceral SMCs (ULTR cells) were grown on either tissue culture (TC)-coated or basement membrane (BM)-coated surfaces. When cells reached >90% confluency, confluent cells were kept for 6 days in culture medium containing either 10% or 2% FBS, respectively. A and B: phase-contrast microphotographs (a, b, d, and e, bar = 200  $\mu$ m; c and f, bar = 100  $\mu$ m) (A) and double-immunofluorescence staining (bar, 10  $\mu$ m) (B) showing  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) and smooth muscle protein 22- $\alpha$  (SM22 $\alpha$ ) expression in visceral SMCs cultured on TC-coated (a-c) and BM-coated (d-f) surfaces. Nuclei are stained in blue by DAPI.

with differences in the (epi)genetic background of the donor, this greatly hampers reproducibility of studies. Our use of established immortalized SMC lines ensures a consistent supply of cells with similar properties while also reducing practical logistic issues associated with isolating cells from tissue.

Vascular and visceral SMCs have been shown to display a substantially different response to changes in microenvironmental stimuli (11, 12, 27). In addition, they express different actin isoforms (39). Whereas  $\alpha$ -SMA is the classical marker of early vascular SMC differentiation (25),  $\gamma$ -SMA is the dominant actin isoform in visceral SMCs (esophagus, stomach, intestine, bladder, and uterus) (1). Although we observed an increased  $\alpha$ -SMA expression in confluent visceral SMCs grown on a regular tissue culture-treated growth surface,

$\gamma$ -SMA responded particularly strongly to the addition of basement membrane matrix and TGF- $\beta$ 1. This may indicate that the optimized culture conditions described here more closely resemble the in vivo visceral SMC microenvironment, which is associated with relatively higher  $\gamma$ -SMA expression.  $\gamma$ -SMA and other SMC contractile genes like SM22 $\alpha$ , smoothelin, and caldesmon are transcriptionally regulated by SRF and its muscle-restricted cofactor myocardin (10, 41). Disruption of the SRF-myocardin complex in vascular SMCs results in an overall decrease of contractile marker proteins (40). In line with this, loss of myocardin causes profound defects in the vasculature. Since the gastrointestinal and genitourinary tracts are also affected in myocardin knockouts, myocardin is thought to be required in both vascular and visceral SMCs (17).

Fig. 5. Controlled modulation of the visceral smooth muscle cell (SMC) phenotype by PDGF-BB and transforming growth factor  $\beta$ 1 (TGF- $\beta$ 1). Visceral SMCs (ULTR cells) were cultured for 6 days with 2% FBS to induce the expression of contractile SMC markers. After 6 days, cells were incubated with either PDGF-BB (30 ng/ml) or TGF- $\beta$ 1 (5 ng/ml). **A**: protein expression levels of smooth muscle myosin heavy chain (SM-MHC),  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA), and smooth muscle protein 22- $\alpha$  (SM22 $\alpha$ ) were determined by Western blot analysis after 48 h. **B** and **C**: mRNA expression levels of contractile (*ACTA2*, *ACTG2*, *TAGLN*, *SMTN*, *CALD1*, and *MYH11*) (**B**) and transcriptional regulatory (*SRF*, *MYOCD*) (**C**) genes were determined after 24 h. mRNA expression levels are expressed as relative expression levels with untreated samples set at 1. Data were normalized to *CYPA* and *B2M* reference genes. Results were obtained from two (*CALD1* and *MYH11*;  $n = 6$ ) or three (*ACTA2*, *ACTG2*, *TAGLN*, *SMTN*, *SRF*, and *MYOCD*;  $n = 9$ ) independent experiments (mean  $\pm$  SE, Kruskal-Wallis test followed by Dunn's post hoc testing with Bonferroni correction;  $*P < 0.05$ ). *ACTA2*,  $\alpha$ -smooth muscle actin; *ACTG2*,  $\gamma$ -smooth muscle actin; *B2M*,  $\beta$ -2-microglobulin; *CALD1*, caldesmon; *CYPA*, cyclophilin A; *MYH11*, smooth muscle myosin heavy chain; *MYOCD*, myocardin; *SMTN*, smoothelin; *SRF*, serum response factor; *TAGLN*, smooth muscle protein 22- $\alpha$ .



Our results confirm expression of SRF and myocardin in uterine SMCs in vitro and show that their levels increase in parallel with contractile SMC markers when confluent visceral SMCs are cultured on BM matrix. Importantly, we observed only low expression of SRF and myocardin in SMCs cultured on tissue culture-treated plastic.

The basement membrane that surrounds SMCs in vivo consists of laminin, collagen IV, heparin sulfate proteoglycan, entactin, and various growth factors (i.e., fibroblast growth factor, TGF- $\beta$ 1, epidermal growth factor, etc.). When SMCs are isolated from tissues, this extracellular environment is lost, and contractile and cytoskeletal filaments are reorganized to adapt to a two-dimensional culture environment, resulting in the acquisition of a synthetic phenotype (35). However, reintroduction of basement membrane components like laminin and collagen IV has been shown to promote the contractile phenotype in vascular SMCs (14, 20, 35, 37). To mimic the in vivo microenvironment associated with the contractile phenotype in culture, we used a growth factor-reduced basement membrane matrix (Geltrex) that is purified from the Engel-

breth-Holm-Swarm tumor. The composition of this matrix is similar to the basal lamina that surrounds SMCs in vivo (19). Since we observed marked induction of all contractile markers and their transcriptional regulators upon culturing human uterine SMCs on a basement membrane-coated growth surface, components of the basement membrane matrix appear to induce a contractile phenotype in visceral SMCs as well. Furthermore, the stiffness of the basement membrane-coated surface is likely to be different from that of regular uncoated surfaces used to culture cells, which also affects the SMC phenotype. For example, SMCs are known to proliferate more quickly on stiff compared with soft substrates, indicating a synthetic phenotype (29). In line with this, we found that the relatively soft surface provided by the basement membrane coating was associated with a more contractile phenotype.

Withdrawal of FBS from the culture medium is a commonly used method to initiate differentiation of many different cell types, including vascular SMCs (18, 21, 43). For example, Solodushko and colleagues (34) showed that confluent serum-starved vascular SMCs respond to increased serum concentra-



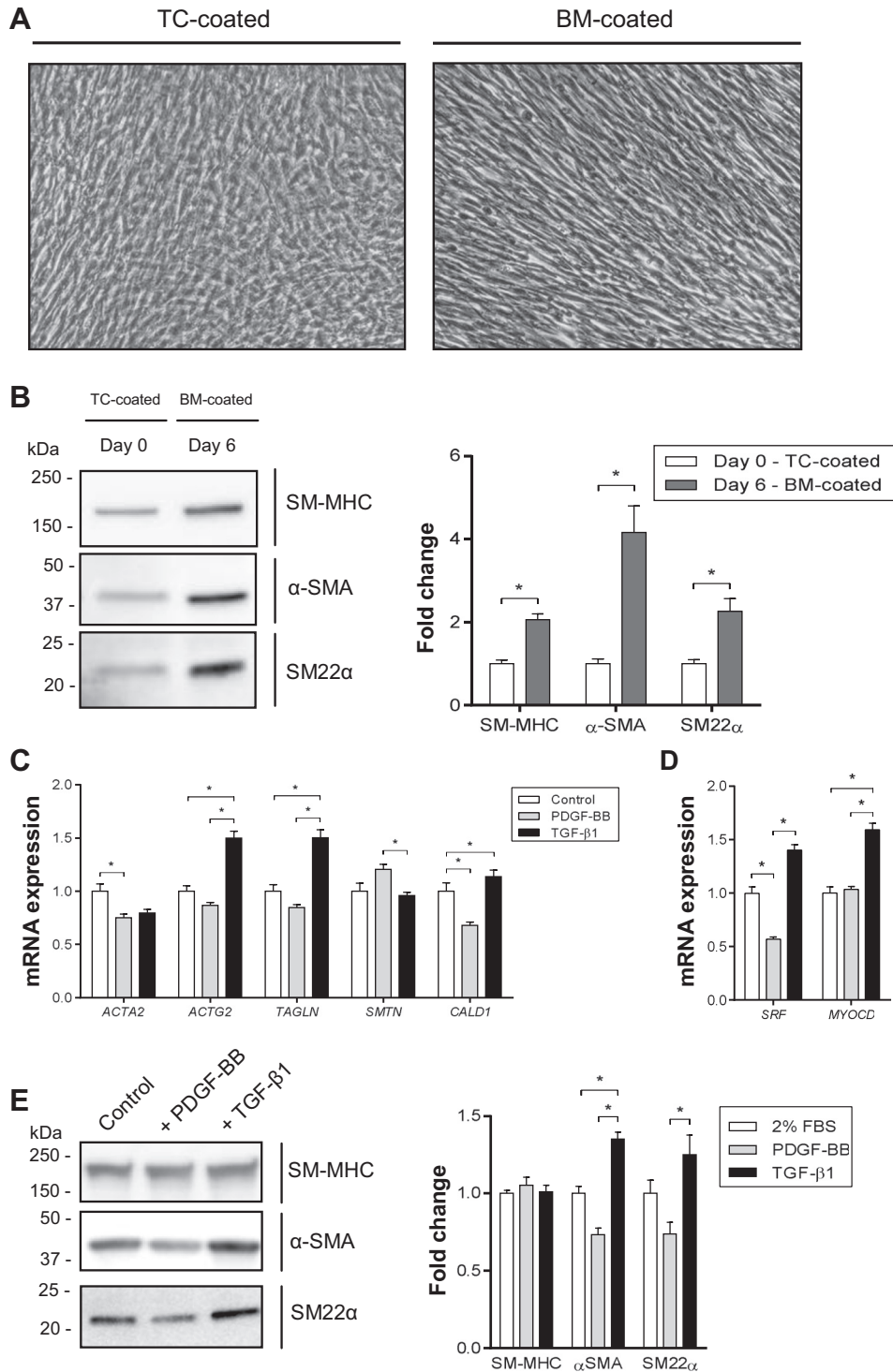


Fig. 6. Validation of culture conditions for controlled phenotypic modulation of visceral smooth muscle cells (SMCs) using telomerase-immortalized human myometrial cells (hTERT-HM). Human hTERT-HM SMCs were grown on either tissue culture (TC)-coated or basement membrane (BM)-coated surfaces. When cells reached >90% confluency (day 0), confluent cells were kept for 6 days in culture medium containing either 10% or 2% FBS, respectively. **A**: phase-contrast microphotographs. Confluent SMCs grown on TC-coated surfaces (left) and confluent SMCs grown on BM-coated surfaces (right) at day 6. **B**: protein expression levels of smooth muscle myosin heavy chain (SM-MHC),  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA), and smooth muscle protein 22- $\alpha$  (SM22 $\alpha$ ) (**B**) expressed by hTERT-HM SMCs cultured on either TC-coated surfaces (day 0) or BM-coated surfaces (day 6). Protein expression levels were determined by Western blot analysis. All samples from each independent experiment ( $n = 3$ , each with biological triplicates) were derived at the same time and processed in parallel. Results are presented as mean  $\pm$  SE (Mann-Whitney  $U$ -test,  $*P < 0.05$ ). **C** and **D**: mRNA expression levels of contractile (*ACTA2*, *ACTG2*, *TAGLN*, *SMTN*, and *CALD1*) (**C**) and transcriptional regulatory (*SRF*, *MYOCD*) (**D**) markers were determined in hTERT-HM SMCs treated with PDGF-BB and TGF- $\beta$ 1 (24 h). Results are expressed as relative expression with untreated samples set at 1. Data were normalized to *CYPB* and *B2M* reference genes and are presented as mean  $\pm$  SE. **E**: protein expression levels of SM-MHC,  $\alpha$ -SMA, and SM22 $\alpha$  were determined by Western blot analysis after hTERT-HM were treated for 48 h with either PDGF-BB or TGF- $\beta$ 1. (Kruskal-Wallis test followed by Dunn's post hoc testing with Bonferroni correction;  $*P < 0.05$ ). *ACTA2*,  $\alpha$ -smooth muscle actin; *ACTG2*,  $\gamma$ -smooth muscle actin; *B2M*,  $\beta$ -2-microglobulin; *CALD1*, caldesmon; *CYPB*, cyclophilin A; *MYOCD*, myocardin; *SMTN*, smoothelin; *SRF*, serum response factor; *TAGLN*, smooth muscle protein 22- $\alpha$ .

tions with increased cell cycle progression and increased DNA synthesis, indicating that serum factors overrule contact inhibition in confluent vascular SMCs. Similar results were reported by Kato and colleagues (18), who showed that high FBS concentrations promoted cell cycle progression and increased total protein and collagen synthesis rates in comparison to low serum conditions in postconfluent rat aortic SMCs. Along this line, we expected that serum deprivation would promote a shift toward the contractile phenotype. However, reducing serum

concentrations had only minimal effects on the expression of contractile markers in visceral SMCs cultured on basement membrane matrix. This may be explained by the fact that basement membrane matrix proteins inhibit growth factor-induced proliferation and, therefore, inhibit the shift toward the synthetic phenotype (9, 16).

Previous studies have shown that cell-cell contact induces growth arrest of vascular SMCs, which subsequently results in the induction of smooth muscle specific contractile proteins (4,

18, 26). Even in the presence of 10% FBS, density-induced growth arrest has been shown to be associated with elevated  $\alpha$ -SMA expression in vascular SMCs (18, 26). In contrast to this, we only observed a minimally increased expression of contractile protein markers and their transcriptional regulators in visceral SMCs kept confluent for 6 days. This indicates that visceral SMCs are less responsive to the increases in cell-cell interactions in the postconfluent state compared with vascular SMCs.

A limitation of our culture model is the absence of non-muscle cells that regulate the contractile functionality of SMCs, such as neuronal cells and the interstitial cells of Cajal. Furthermore, it should be noted that we cannot distinguish between cell hypertrophy and increased cell proliferation as a cause of smooth muscle marker protein differences between the different culture conditions. In addition, immortalized cell lines can acquire different characteristics compared with normal cells due to activation of different gene expression programs. Nevertheless, SMC plasticity remained intact in our model since contractile marker expression could be both induced and repressed.

In conclusion, we developed a novel visceral SMC culture system in which confluent visceral SMCs can be kept in a contractile phenotype by culturing on a BM coating under low serum conditions. This contractile phenotype can be further modulated toward a more contractile or a more synthetic state by TGF- $\beta$ 1 or PDGF-BB stimulation. As such, this SMC culture system represents a highly suitable model to study phenotypic modulation of visceral SMCs in health and disease.

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#### DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

#### AUTHOR CONTRIBUTIONS

R.D.W.V., S.W.M.O.D., and S.S.R. conceived and designed research; R.D.W.V., L.v.d.B., and B.B. performed experiments; R.D.W.V. and D.P.J.v.D. analyzed data; R.D.W.V. and S.S.R. interpreted results of experiments; R.D.W.V. prepared figures; R.D.W.V. drafted manuscript; R.D.W.V., L.v.d.B., D.P.J.v.D., S.W.M.O.D., and S.S.R. edited and revised manuscript; R.D.W.V., L.v.d.B., B.B., D.P.J.v.D., S.W.M.O.D., and S.S.R. approved final version of manuscript.

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